Reference: *Biol. Bull.* **193:** 116–124. (October, 1997)

Catecholamine-Containing Cells in Larval and Postlarval Bivalve Molluscs

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Abstract. Previous studies have suggested an involvement of catecholamines in the control of several larval behaviors, such as feeding, locomotion, and induction of settling and metamorphosis. In the present study we employed aldehyde-induced, blue-green fluorescence to indicate catecholamines in cells within representatives of two bivalve families, the Pectinidae (*Placopecten magellanicus*) and the Mytilidae (*Mytilus edulis*). Larvae were examined at different stages of development before and also shortly after settlement. The general distribution of fluorescent cells was similar in the two species. By mid-veliger stage, several fluorescent cells and fibers were located along the outer rim of each velar lobe, and a pair of flask-shaped cells was located lateral to the mouth. A single fiber from near the mouth projected to a region beneath the apical tuft. In the pediveliger, the cells by the mouth were joined by an additional two to four fluorescent cells. The developing foot also contained numerous such cells, some of which had processes that penetrated the epithelium on the "sole" and bore ciliated terminals. Fluorescent somata were also located around the edge of the mantle. Centrally projecting fibers appeared to terminate in the pedal and abdominal ganglia, which also contained a few fluorescent somata. After settlement, the velar lobes and resident fluorescent somata disappeared, but fluorescent cells in the foot persisted as this latter organ grew. Fluorescent cells within the developing gill were connected with the abdominal ganglia by means of fibers. Control preparations labeled with antibodies raised against serotonin indicated that the aldehyde-induced fluorescence was not due to the presence of indoleamines. The present study not only confirms previous chromatographic evidence suggesting the presence of catecholamines in the larvae of bivalve molluscs, but also identifies putative neuronal circuits that may control various larval behaviors.

Received 22 January 1997; accepted 24 July 1997.

Introduction

Catecholamines have been implicated in many of the physiological processes that occur during the larval development of molluscs. For instance, several lines of evidence suggest that these substances can act as morphogens, directly influencing early development (Voronezhskaya *et al.*, 1992, 1993; Buznikov *et al.*, 1996). Other work suggests roles for catecholamines in the control of ciliary activity (Beiras and Widdows, 1995a) and in the triggering of settling and metamorphosis (Hadfield, 1984; Coon *et al.*, 1985; Voronezhskaya *et al.*, 1992, 1993; Beiras and Widdows, 1995b; but see Pires and Hadfield, 1991). Although chromatographic techniques have indicated the presence of catecholamines in molluscan larvae (Coon and Bonar, 1986), the catecholaminergic neurons and pathways, which have been hypothesized to mediate such processes (Bonar *et al.*, 1990; Beiras and Widdows, 1995a, b), have not previously been identified. In the present report we provide histochemical evidence that many catecholaminergic neurons are located in the velum, foot, mantle, central nervous system, and mouth area of mussel and scallop larvae, and we describe how the distributions of these neurons change with metamorphosis. This work thus provides new foci for the critical testing of hypotheses about the roles of catecholamines in the development of molluscan larvae and provides a basis for comparisons with parallel functions across related phyla (Marsden and Hassessian, 1986; Edwards *et al.*, 1987; Hay-Schmidt, 1990a, b, 1992).

Materials and Methods

Animals

Adult blue mussels (*Mytilus edulis*) were collected from the Aqua Prime aquacultural farm in Ship Harbour, Nova Scotia, and transferred to the Aquatron Seawater Laboratory of Dalhousie University. Spawning was induced by thermal stimulation (8°–10°C above ambient), and the larvae were incubated in 50-l polyethylene containers for 72 h at 12°C until the straight-hinged (D-stage) veliger was reached. (See Bayne, 1971, for a description of larval development in *M. edulis*.) Larvae were then transferred to 1000-l insulated tanks filled with 12°C, filtered (0.2 μm) seawater, which was changed every 2 or 3 days. The algal food source was *Isochrysis galbana* (clone ISO; Provasoli-Guillard Center for Culture of Marine Phytoplankton, West Boothbay Harbor, ME), fed at a concentration of 25,000 cells/ml after each water change. Larvae of the sea scallop (*Placopecten magellanicus*) were obtained directly from the Fisheries Resource Development Corporation hatchery in Sandy Cove, Nova Scotia, and were prepared for histochemical study immediately after their delivery to the laboratory. (See Culliney, 1974, and Cragg and Crisp, 1991, for descriptions and staging of larval development in *P. magellanicus*.)

Before relaxation or fixation, the larvae were placed under a dissecting microscope to observe their behavior and general morphology.

Histochemistry

Larvae were collected on a 60-μm-mesh Nitex screen and rinsed into 20-ml vials with filtered (0.2 μm) seawater. Whole larvae were either first relaxed by gradually adding 7.5% (W:V) $MgCl_2$ to their natural seawater bath until the valves gaped open and the vela were extended (Omori and Ikeda, 1984) or were processed directly using the FaGlu procedure modified from Furness *et al.* (1977). In brief, living larvae were immersed in a solution of 4% paraformaldehyde and 0.55% glutaraldehyde in phosphate-buffered saline (PBS: 50 m*M* $Na_2HPO_4 \cdot 7H_2O$ and 140 m*M* NaCl, pH 7.4). After 2–18 h, the fixed larvae were placed on glass slides, excess solution was removed with paper wicks, and the slides were dessicated in the dark at room temperature for 4–18 h. A drop of either paraffin oil or methyl salicylate was added before a coverslip was placed over the slide. The mounted slides were viewed through a Leitz Aristoplan microscope equipped for ultraviolet (UV) fluorescence (D filter block providing 355–425 nm excitation and 460 nm long-pass barrier filter) and photographed using either Kodak T-MAX 100 or Ektachrome 400 film. Negative controls were also prepared with no glutaraldehyde added to the fixative. Such preparations exhibited none of the blue-green fluorescence reported in this study.

Similar histochemical techniques are known to specifically induce blue-green fluorescence that is characteristic of catecholamines in a wide variety of animals (Schöler and Armstrong, 1982; Hauser and Koopowitz, 1987; Molist *et al.*, 1993), including molluscs (Croll and Chiasson, 1990). Little or no yellow fluorescence of indoleamines (Furness *et al.*, 1977) was noted in any of the aforementioned studies. To further exclude the possibility that indoleamines contributed to the fluorescence patterns observed in the study, we also labeled several ($n \approx 50$) pediveligers of *M. edulis* with antibodies raised against serotonin conjugated to bovine serum albumin with paraformaldehyde. Procedures were modified from Marois and Croll (1992) and Marois and Carew (1997). Thirty-four-day-old *M. edulis* larvae were fixed overnight in 4% paraformaldehyde in PBS. The embryos were then transferred to 70% ethanol in which they were stored at −20°C. For histology, the stored larvae were washed briefly in PBS and then placed into 10% EDTA in PBS for 20 min at room temperature. After another brief rinse in fresh PBS and overnight incubation in 4% Triton X-100 in PBS, the larvae were transferred into a 1:500 dilution of rabbit anti-serotonin antibodies (Incstar, Stillwater, MN) in PBS for 48–72 h. These latter and all subsequent steps were performed at 4°C. Larvae were then washed in PBS for 6 h before incubation overnight in 1:50 dilution of FITC-labeled goat anti-rabbit antibodies (Bio/Can Scientific, Missassauga, Ontario). Larvae were then washed in PBS and mounted in 3:1 glycerine in PBS. Specimens were viewed using an L3 filter cube (permitting 450–490 nm illumination and visualization through a 525/20 nm bandpass filter) on the Leitz microscope and were photographed using Kodax T-MAX 100 film. Specimens prepared according to these same procedures but omitting the primary antibody showed none of the fluorescence reported below.

Results

At 22 days of age, the veligers of *P. magellanicus* (about 180 μm shell length, as measured along the major axis) possessed neither eye spots nor feet. Blue-green fluorescence, indicative of catecholamines, was located within cells in the velum (Fig. 1A) and around the mouth (Fig. 1B). Each lobe of the velum contained two to four fluorescent cell bodies, which were generally shaped as squat cones and evenly spaced in a line along the outer rim (Fig. 2A, B). Two to three small fibers, each possessing several varicosities, could be seen running between adjacent cells along each rim (Fig. 2A–C), while other fibers coursing between the most dorsal cells connected the two velar lobes. A pair of flask-shaped, fluorescent cells was located just lateral to the mouth of the veliger (Fig. 2D). A stout apical process was directed

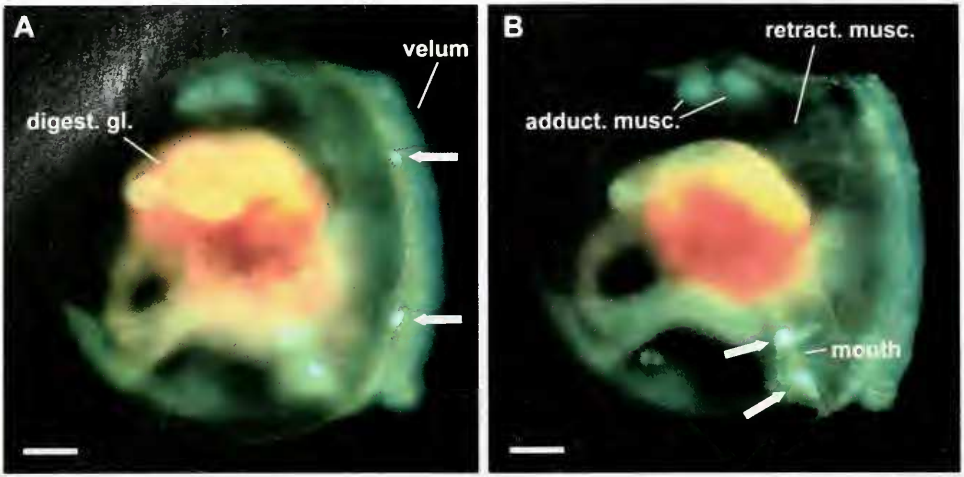

Figure 1. Wholemounted, 22-day-old *Placopecten magellanicus* veliger viewed at two focal planes. (A) The partially retracted velum is to the right, and the digestive gland (digest. gl.), which exhibits red autofluorescence, lies toward the left, near the valve hinge. Aldehyde-induced, blue-green fluorescence is localized to two cells (arrows) and interconnecting fibers along the outer rim of the velum. (B) Single, blue-green fluorescent, flask-shaped cells (arrows) are also located on each side of the mouth. Velar retraction has displaced one of the cells slightly ventrally in this figure. The faint outlines of valve adductor muscles (adduct. musc.) and velar retractor muscle (retract. musc.) can also be seen. Calibration bars in A and B equal about 23 μm.

dorsolaterally from each of these cell bodies, and thin fibers running across the midline immediately ventral to the mouth interconnected the two somata. Generally, another single fiber could be seen running between the mouth region and an area directly behind the apical tuft at the very center of the velum (Fig. 2A; see Raineri, 1995).

By 36 days of age, the larvae of *P. magellanicus* (about 275 μm shell length) possessed prominent feet and occasionally one or two gills. The general pattern of staining in the vela of these pediveligers was essentially the same as that seen in 22-day-old larvae, although the vela were smaller in such specimens (Fig. 3C). The foot of each pediveliger contained 15–20 fluorescent cells located predominantly toward the tip and along the ventral side (sole) of the appendage (Fig. 3A–C). Distal processes from the subepithelial somata of at least some of these cells appeared to bear long cilia that projected from the surface of the foot (Fig. 3B). Axons from the fluorescent cells of the foot appeared to project centrally and converge upon the developing pedal ganglia (Fig 3C), although such details were often partially obscured by the thicker shells and body tissues of these and older larvae. These larvae also possessed one to two additional pairs of fluorescent cell bodies near the mouth, three to four cell bodies located within the abdominal ganglia, and a few cells along the edge of the mantle (Fig. 3C).

At 31 days, the larvae of *M. edulis* (about 255 μm shell length) generally resembled the pediveligers of *P. magellanicus* both in general morphology and in distribution of blue-green fluorescent cells and fibers. For example, each velar lobe contained a string of three to five fluorescent cells along its outer rim (Fig. 3D). Another four to six cell bodies were clustered near the mouth, four to five cell bodies were located within the abdominal ganglia, and 25–30 cell bodies were located within the foot, often with a single isolated soma near the heel. Prominent fiber tracts connected both the cells in the foot with the pedal ganglia and the pedal ganglia with the abdominal ganglia. In addition, a few cells and fibers were occasionally detected along the edge of the mantle. (See Fig. 4A for a schematized summary of the distribution of fluorescent elements in less developed pediveliger larvae of *M. edulis*.)

At 34 days, most of the larvae of *M. edulis* (shell size remained the same as in younger specimens) exhibited signs of settling. A large percentage of the larvae crawled along the substratum using enlarged feet that were subsequently revealed to contain 30–40 fluorescent cell bodies. The vela of these larvae were smaller than those of the

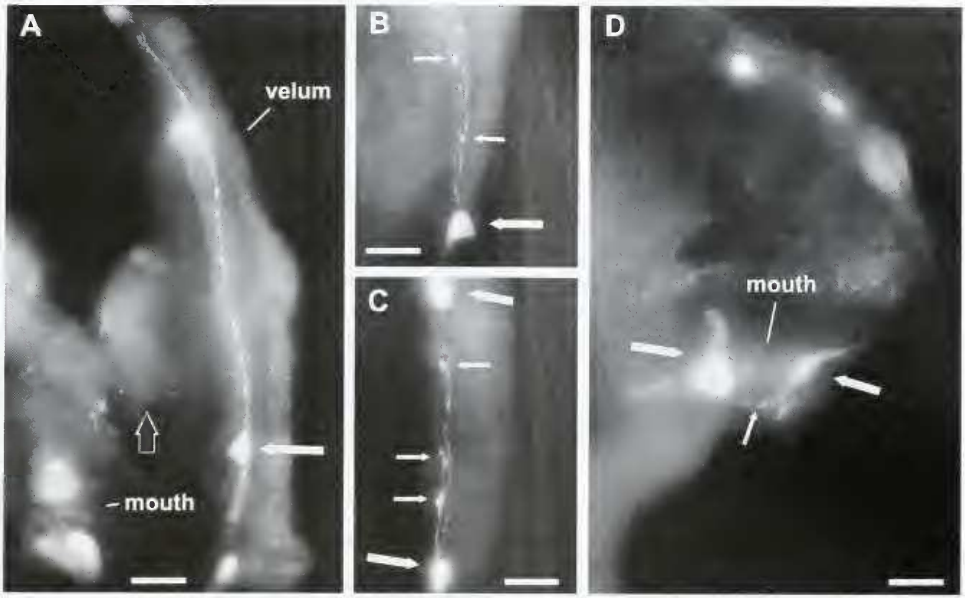

Figure 2. Aldehyde-induced fluorescence in 22-day-old veligers of *Placopecten magellanicus*. (A) Solid arrow indicates a squat, conical cell along a string of fibers on the rim of the velum. One to two fluorescent cells (out-of-focus) are located on each side of the mouth. Unfilled arrow indicates a small fiber that originates near the mouth and then terminates in what may be the apical ganglion located in the middle of the velum. (B) This shows another example of the squat conical shape of fluorescent velar cells (larger arrow). Fibers appear to exit at the base of the cell. Numerous varicosities (smaller arrows) are found along the lengths of the fibers. (C) In many cases two to three fibers with varicosities (smaller arrows) interconnect adjacent fluorescent cells (large arrows) in the velum. (D) Flask-shaped cells (larger arrows) and interconnecting fibers (smaller arrow) are found around the mouth. Calibration bars in A, B, C, and E equal about 12 μm.

younger larvae, but strings of three to four fluorescent cells and interconnecting fibers could still be detected along each of the outer rims (Fig. 4A). Single fluorescent cells were also located at the tips of each of the two to three gill bars of these larvae, and a fiber tract could be detected connecting the most posterior gill arch with the abdominal ganglia (Fig. 3E). As with *P. magellanicus*, visualization of fluorescence in such larvae was often obscured by the shell and growing body tissues.

By 41 days, most *M. edulis* either had vela that were further reduced in size (Fig. 4B) or had completely lost their vela. Although the shell length changed little from earlier stages, new growth resulted in broader, less elliptical shells, indicating the addition of postmetamorphic dissoconch (Bayne, 1971). Prominent cells could still be seen around the mouth and within the abdominal ganglia and foot of each animal. Several of these specimens also contained fluorescent byssal fibers running along the anterodorsal margin of the foot from its base to apex. The gills contained a total of four to five arches, each with one to two fluorescent cells. (See Fig. 4B for a schematic summary of the distribution of fluorescent elements in post-settling specimens of *M. edulis*.)

Immunocytochemical labeling of serotonin produced a completely different staining pattern than that induced by aldehydes in 34-day-old *M. edulis*. Compare Figure 5A, B (aldehyde-induced fluorescence) with Fig. 5C–F (serotonin-like immunoreactivity). The velum contained no intrinsic serotonin-like immunoreactive somata but only varicose fibers (Fig. 5C–E), which appeared to originate from three somata located near the midline just behind the center of the velum (Fig. 5E). Likewise, the foot contained no intrinsic immunoreactive somata but only varicose fibers (Fig. 5C-E), which appeared to originate

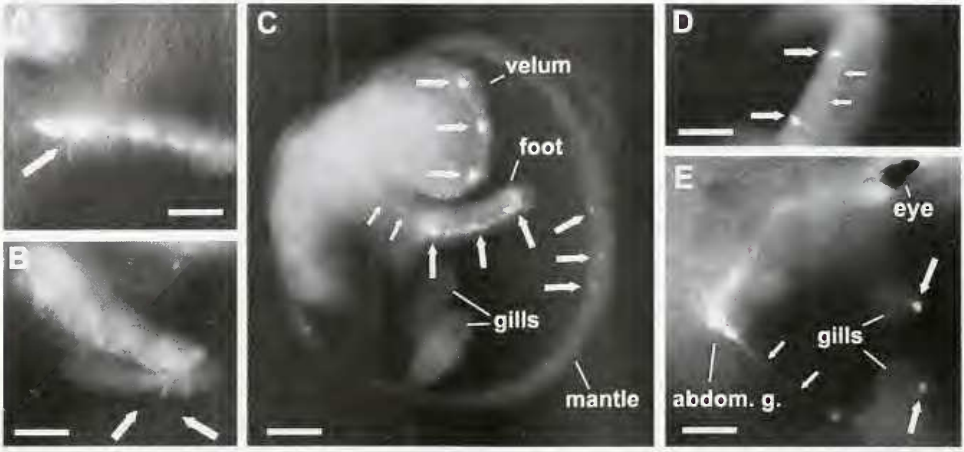

Figure 3. Aldehyde-induced fluorescence in pediveligers of *Placopecten magellanicus* and *Mytilus edulis*. (A) A number of fluorescent cells in the foot of a 36-day-old *P. magellanicus* pediveliger possess processes (arrow) that extend from subepithelial somata to the ventral surface of the foot. (B) At least some of these processes appear to bear long cilia (arrows). (C) A 36-day-old pediveliger of *P. magellanicus* with gills. Larger arrows indicate three cells in the velum, three cells in the mantle, and numerous cells in the foot. Smaller arrows indicate fluorescent fibers projecting to the pedal ganglia (not in focus). (D) Fluorescent somata (larger arrows) and fibers (smaller arrows) along the rim of the velum of a 31-day-old pediveliger of *M. edulis*. (E) A 34-day-old veliger of *M. edulis* with a prominent eyespot (eye). Larger arrows indicate cells in two gills. Smaller arrows indicate fluorescent fibers connecting gills with abdominal ganglia (abdom. g.). Calibration bars equal about 21 μm in A and B, 38 μm in C, about 30 μm in D, and about 17 μm in E.

from cells in the developing pedal ganglia. Varicose fibers innervating the mantle appeared to originate from the abdominal ganglia (Fig. 5F).

Discussion

The distribution of fluorescent cells described in this report is consistent with both chromatographic evidence (Coon and Bonar, 1986) indicating the presence of catecholamines in bivalve larvae and evidence from other studies suggesting major roles for catecholamines in such important functions as locomotion, feeding, and the triggering of settling behavior and metamorphosis (Bonar *et al.*, 1990; Beiras and Widdows, 1995a, b). However, although the presence of some catecholamine-containing cells may have been expected, the locations and relatively large numbers of neurons described in this study are surprising in light of the commonly held belief that the nervous system of molluscs is rudimentary until relatively late larval stages (Raven, 1966; Kandel *et al.*, 1981; Moor, 1983). To date, most work on molluscan neural development has focused upon embryonic cells resident within the developing central ganglia (Kempf *et al.*, 1987, 1992; Marois and Carew, 1990; Marois and Croll, 1992; Barlow and Truman, 1992). Indeed, our study also permitted the identification of small numbers of cells in central ganglia. Aldehyde-induced fluorescence and serotonin-like immunoreactivity were present in different cell bodies and converging fibers in a region immediately behind the center of the velum, a region in which the apical or cerebral ganglion has previously been described in larval bivalves (Bayne, 1971; Raineri, 1995). Our study also identified cell bodies and fibers in the previously described developing pedal and abdominal ganglia (Bayne, 1971; Raineri, 1995). However, our work additionally demonstrates that many more catecholaminergic neurons are located peripherally and may play significant roles in the control of the velum, foot, mouth, and mantle and, in later larvae, also the gills. Furthermore, similarities between the neurons found in *P. magellanicus* and *M. edulis*, which respectively represent the Pectinidae and Mytilidae families of bivalves, suggest that the distribution of catecholamine-containing neurons may be generalized more widely across this taxon. Very similar distributions of catecholaminergic neurons have recently been found in a variety of gastropod larvae as well (E. E. Voronezhskaya, L. Hiripi, K. Elekes, and R. P. Croll, unpubl. data), thus suggesting further generalizations across molluscan classes.

The goal of the present research was to document the

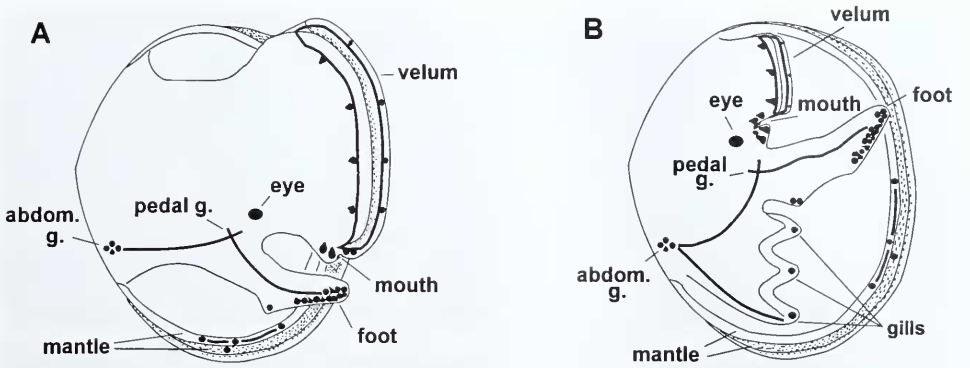

Figure 4. Distribution of aldehyde-induced fluorescent elements in *Mytilus edulis* before and after settlement. (A) Young pediveliger with eyespots (eyes). Larva contains various fluorescent cells and fibers in the velum, foot, and mantle and around the mouth. Fluorescent fibers connect the pedal ganglia (pedal g.) with cells in the foot and in the abdominal ganglia (abdom. g.). (B) After settlement the foot grows while the velum shrinks in size. Fluorescent cells are added to the developing gills, which are connected to the abdominal ganglia by means of fluorescent fibers. Lengths of major shell axes in both A and B equal about 255 μm.

presence and describe the distributions and projections of catecholamine-containing neurons in the larvae of bivalve molluscs. In the course of this work we examined specimens at the veliger and pediveliger stages of larval ontogeny and also at settlement and metamorphosis into juvenile stages. Nothing is currently known about the developmental origins of such cells, although such a study would follow one obvious avenue for future research. The present study does, however, offer unique insights into later larval life and metamorphosis. Much of the work on the neural changes associated with molluscan metamorphosis has focused upon the velum (Marois and Carew, 1990, 1997; Barlow and Truman, 1992), which had previously only been reported to receive innervation originating from the cerebral ganglia (see also Arkett *et al.*, 1987; Mackie *et al.*, 1976; but see Marois and Carew, 1997). These previous studies indicated that both the axons and originating somata disappear as the velum is lost. Our control preparations, which revealed serotonin-like immunoreactivity in premetamorphic larvae, exhibited patterns strikingly similar to those previously described in gastropods (see also Croll and Voronezhskaya, 1995; Marois and Carew, 1997); although examination of developmental changes in immunoreactivity was outside the scope of the present study, one might reasonably hypothesize that gastropods and bivalves share similarities in this regard as well. Our observations do indicate, however, that several peripheral neurons also disappear during metamorphosis. Whether the disappearance of catecholamine-containing cells in the velum is due to cell death, changes in transmitter phenotype, or migration must be answered by future research.

By late larval life the foot is a complex structure used for locomotion and byssal secretion and attachment (Lane and Nott, 1975). Its motility and anterior location in the crawling pediveliger also make it ideally suited as a sensory probe. We found that the foot of the pediveliger contains relatively large numbers of catecholaminergic cells. At least some of these cells appear to have apical processes that traverse the epithelium and bear long cilia projecting from the "sole" of the foot. Similar ciliated cells have long been thought to mediate chemoreception and mechanoreception in molluscs (Croll, 1983). Furthermore, these peripherally located somata are connected to the pedal ganglia *via* fiber pathways and might therefore involve not only local control of the foot but also centrally mediated functions. Since development of the foot is acknowledged to be a necessary precondition for metamorphosis in molluscs (Bonar, 1978; see also Bayne, 1971, Culliney, 1974), it is tempting to hypothesize that metamorphic competence results from the maturation of catecholaminergic pathways in the foot.

Consistent with our observations on larvae, both catecholamines and serotonin have long been known to be present and active in adult bivalves (Greenberg, 1960; Sweeney, 1968). More recent work confirms that such monoamines are, in fact, widespread within the tissues of bivalves such as *P. magellanicus* (Croll *et al.*, 1995; Pani and Croll, 1995). Particularly relevant to this study is the finding (Smith, 1996; S. A. Smith, J. Nason, and R. P.

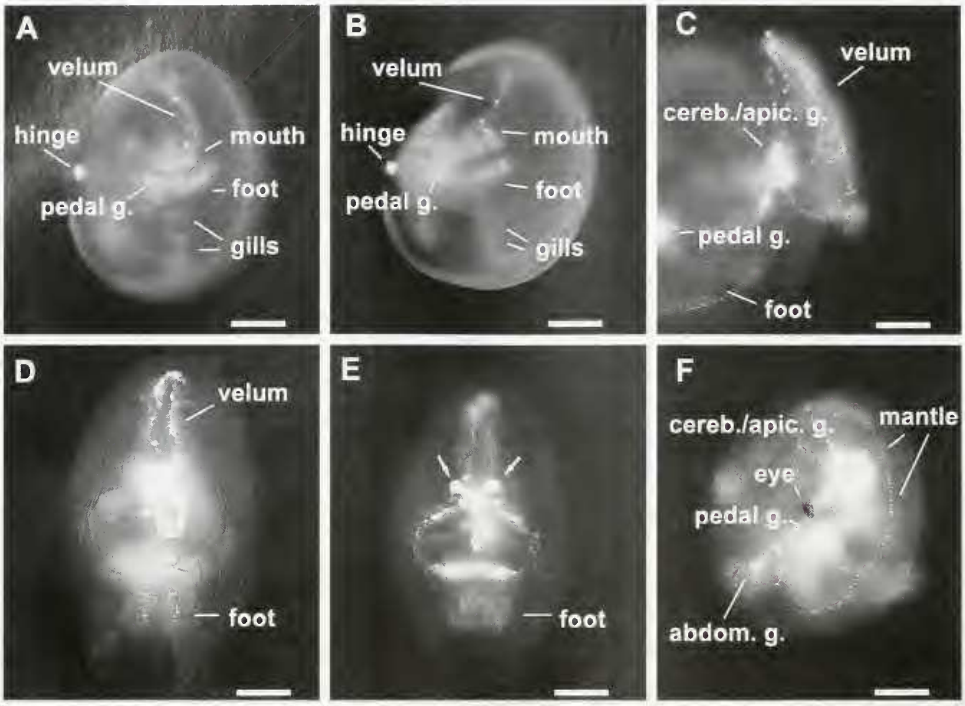

Figure 5. Aldehyde-induced fluorescence of catecholamines (A, B) and serotonin-like immunoreactivity (C-F) in pediveligers of *Mytilus edulis*. (A) Aldehyde-induced fluorescence in a 34-day-old larva with gills. A string of somata can be seen along the outer rim of the velum. Although the developing shell and body tissues often obscure details, diffuse fluorescence from numerous out-of-focus cells can be seen along the entire foot and in a concentration in the pedal ganglia (pedal g.). The hinge of *M. edulis* autofluoresces yellow whether or not glutaraldehyde is added to the fixative. (B) The velum is much smaller in this 41-day-old specimen, although aldehyde-induced fluorescence can still be seen in cells along its outer rim. Other cells can be seen in the region of the mouth and the foot. (C) Serotonin-like immunoreactivity in a 34-day-old pediveliger. Varicose fibers in the velum appear to originate from somata in the underlying cerebral or apical ganglia (cereb./apic. g.). Varicose fibers in the foot appear to originate from somata in the pedal ganglia. (D) Frontal view shows varicose serotonin-like immunoreactive fibers along the outer rim of the velum and within the foot. (E) A deeper focus shows that one medial (larger arrow) and two lateral (smaller arrows) serotonin-like immunoreactive somata in the cerebral or apical ganglia underlie the velum. (F) Lateral view shows varicose fibers running around the edge of the mantle. Immunoreactive somata or converging fibers clearly mark the locations of the developing cerebral or apical ganglia, pedal ganglia, and abdominal ganglia (abdom. g.). Calibration bars equal about 58 μm in A, B, and F and about 38 μm in C, D, and E.

Croll, unpubl. data) that adult bivalves possess catecholamine-containing neurons that reside not only within the central ganglia but also peripherally, where they are particularly concentrated in the foot and gills and surrounding the mouth, as was observed here in specimens at around the time of metamorphosis.

As the first report detailing the distribution of catecholaminergic neurons in molluscan bivalve larvae, this study provides new foci for the study of larval form, function, and development. Previously hypothesized neuronal control mechanisms have now been tentatively identified and their actual functions can be critically tested using electrophysiological, pharmacological, and ablation techniques. These findings also suggest that larval behavior and physiology are likely to be affected by interactions between several different neurotransmitters. For example, our his-

tological work indicates that velar activity is probably influenced by both catecholamines and serotonin, as suggested by previous pharmacological studies (Beiras and Widdows, 1995a). The presence of acetylcholinesterase activity suggests that acetylcholine might also play important roles in larval function (Raineri, 1995). The finding of Phe-Met-Arg-Phe-NH_2 (FMRFamide)-like immunoreactivity in early larvae of gastropods (Croll and Voronezhskaya, 1995, 1996) and bivalves (R. P. Croll and E. E. Voronezhskaya, unpubl.) adds yet another layer of complexity to our view of neural functions in molluscan larvae. It is likely that the catalog of probable neuroactive substances will grow as specific labels are increasingly applied to the study of such larvae, and further elucidation of the neural pathways in bivalve larvae will provide a better understanding of the coordination and control of larval locomotion and feeding, and the complex processes of settlement and metamorphosis.

Acknowledgments

Funding was provided by a grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada to R.P.C. and a contract (#9F007-1-8019/01/SR) from the Canadian Space Agency to R. O'Dor and D.L.J. We thank Barry MacDonald of the Fisheries Resource Development, Ltd., for providing scallop veligers, and Kaija Lind of Aqua Prime Mussel Farms for providing mussel broodstock.

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